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Adenovirus-mediated gene transfer of glutamine: fructose-6-phosphate amidotransferase antagonizes the effects of interleukin-1β on rat chondrocytes[1]

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Summary

Objective: To determine whether overexpression of glutamine: fructose-6-phosphate amidotransferase (GFAT) in synoviocytes will antagonize the response to interleukin-1β (IL-1β) of chondrocytes and synovial fibroblasts in co-culture.

Methods: Synovial fibroblasts from the rat were transduced by an adenovirus carrying the cDNA for GFAT and then co-cultured with rat chondrocytes encapsulated in alginate beads. Following challenge with 1, 5, or 10 ng/ml of IL-1β for 24 h, proteoglycan synthesis by the chondrocytes was determined by incorporation of $Na_2^{35}SO_4$. Production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) were monitored by assay of conditioned medium from the co-culture.

Results: IL-1β treatment of untransduced-synoviocyte/chondrocyte co-cultures resulted in markedly decreased proteoglycan synthesis by the chondrocytes, and increased NO and PGE_2 levels in the culture medium. In contrast, adenovirus-mediated transfer of GFAT in synoviocytes prevented both the decrease in chondrocyte proteoglycan synthesis and increases in NO and PGE_2 provoked by IL-1β.

Conclusions: Our study suggests that in a synoviocyte/chondrocyte co-culture system, overexpression of GFAT by synoviocytes significantly inhibits subsequent stimulation by IL-1β *in vitro.* Since GFAT is the rate limiting enzyme in the synthesis of intracellular glucosamine and its derivatives, these results may open new possibilities for osteoarthritis treatment.

Key words: GFAT, Glucosamine, Gene transfer, IL-1.

Introduction

Osteoarthritis (OA), the most common articular disorder, is characterized primarily by the progressive loss of articular cartilage accompanied by sclerosis of subchondral bone and the formation of osteophytes[1]. Although excessive or traumatic joint loading coupled with genetic predisposition are considered initiating factors in OA, many investigators consider interleukin-1 (IL-1) and other inflammatory mediators, synthesized locally by synovial cells and chondrocytes, to be important in the progression of the disease[2,3]. At low levels, IL-1 inhibits proteoglycan and collagen synthesis by chondrocytes, while at higher concentrations, it stimulates increased production of specific tissue degradating enzymes by chondrocytes and synovial fibroblasts[4–6]. These include serine proteases and matrix metalloproteases, such as collagenase, gelatinase, and stromelysin[7]. Moreover, in activated chondrocytes, the pro-inflammatory effects of this cytokine are amplified by a variety of downstream mediators such as prostaglandin E_2 (PGE_2) produced by the induction of cyclo-oxygenase 2, or nitric oxide (NO) formed by inducible nitric oxide synthase (iNOS)[8,9].

Ideally, successful treatment of OA would not only reduce or eliminate joint pain, but would also slow or reverse the loss of articular cartilage. In this respect, present treatments are unsatisfactory. For example, although non-steroidal anti-inflammatory drugs, the most commonly used agents in the management of arthritis, improve the quality of life by diminishing pain and inflammation, they frequently cause adverse side-effects[10,11] and do not modify the course of the degenerative process.

Preliminary evidence suggests that some slow-acting drugs such as glucosamine, may exert a favorable influence on the course of OA[12,13]. Based on their suspected protective and stimulatory effects on cartilage, different formulations, such as glucosamine sulfate and/or chondroitin sulfate, are commercially available and widely touted in the lay press as medications for OA. However, doubts about the efficacy of these agents persist among the medical community. Indeed, despite the fact that glucosamine has been used in clinical trials for more than a decade[14,15], the mechanism by which this amino sugar might mediate its anti-arthritic effects is unknown and has only recently become the focus of basic research[16–20].

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In vitro, the addition of glucosamine to culture medium has been shown to antagonize the effects of IL-1β on chondrocytes[17] and on cartilage explants[21]. In these experiments, though, extraordinarily high concentrations of glucosamine were required to effectively inhibit the responses of chondrocytes to IL-1β. Achieving similar local concentrations *in vivo* would require the administration of unrealistically large quantities of this amino-sugar. It is therefore unlikely that glucosamine ingested orally could attain an equivalent level of efficacy within the joint as it does *in vitro*.

A method by which it may be possible to generate effective levels of glucosamine in the joint tissues is through gene transfer. Although glucosamine cannot be synthesized directly as a gene product, it may be possible to duplicate its effects by increasing the synthesis of glucosamine and glucosamine derivatives in target cells by upregulating the hexosamine pathway. Under normal conditions a small percentage of glucose that enters the cell is shunted through the hexosamine pathway for conversion into precursors of macromolecules based on amino sugars. In the first step of this pathway, fructose-6-phosphate is converted to glucosamine-6-phosphate by the rate limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT)[22]. Glucosamine-6-phosphate is the metabolite into which exogenous glucosamine is first converted following entry into the cell. In recent studies it was shown that the adenoviral-mediated delivery and overexpression of GFAT in pancreatic β-cells induced biological effects similar to those observed from treatment with exogenous glucosamine[23].

Because of their ease of production and application to joint tissues[24,25], recombinant adenoviral vectors were selected as a vehicle for gene delivery for use in this *in vitro* study. Vectors based on this system have been shown to transduce with high efficiency numerous cell types including monolayer cultures of synoviocytes and chondrocytes[26–28]. Unfortunately, efficient transduction of chondrocytes within the dense extra-cellular matrix of cartilage is not presently achievable. Indeed, following direct injection of a recombinant vector, the vast majority of gene transfer occurs within the cells of the synovial lining. Thus, to simulate *in vitro* the process of direct intra-articular gene transfer, we established a system whereby alginate beads seeded with chondrocytes were co-cultured with synovial fibroblasts previously infected with an adenoviral vector carrying the cDNA for GFAT. The responses of the various cell types to IL-1 stimulation were then measured.

The present study was designed to explore the feasibility of this approach as a possible mean of antagonizing the chondrodegenerative effects of IL-1. Our results provide evidence that overexpression of GFAT in synovial cells may achieve a level of protection from IL-1 stimulation similar to that observed previously by addition of glucosamine in culture medium[17].

Materials and methods

RECOMBINANT ADENOVIRUS

The adenoviral vector containing the cDNA green fluorescent protein (Ad.GFP) originated from replication-deficient type 5 adenovirus lacking E1 and E3 loci. The GFP cDNA was inserted into an expression cassette in the E1 region driven by the human cytomegalovirus (CMV) early promoter[29]. To enable expression of both GFP and GFAT from a single adenovirus (Ad.GFAT/GFP)[23], a vector was constructed using the AdEasy system (Stratagene)[30], such that the GFAT coding region was inserted downstream of that for GFP. An internal ribosome entry site contained between the two coding regions permits expression of both gene products from a single polycistronic message. Expression of this construct is also driven by the CMV promoter. Suspensions of recombinant adenovirus were prepared by amplification in 293 cells followed by purification using three consecutive CsCl gradients by established methods[31]. Viral titers were estimated by optical density at 260 nm and standard plaque assay.

CHONDROCYTE ISOLATION AND CULTURE

Articular chondrocytes were isolated from the femoral head caps of Wistar male rats under aseptic conditions (Charles River Laboratories). Chondrocytes were obtained by sequential digestion of the cartilage with pronase and type II collagenase (Life Technologies) as previously described[32] and then cultured in 25-cm^2 flasks in complete Dulbecco's Modified Eagle Medium Ham's F12 ([DMEM/Ham's F-12] supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin [Life Technologies]) at 37°C in a humidified atmosphere containing 5% CO_2. At confluence, the chondrocytes were encapsulated in alginate beads at a concentration of 2×10^6 cells/ml [low viscosity alginate (1.2%, w/v) (from *Macrocystis pyrifera*, Sigma)][33]. Beads were maintained in complete DMEM/Ham's F-12 for 10 days in a humidified atmosphere with 5% CO_2 at 37°C before further experiments.

SYNOVIOCYTE ISOLATION AND CULTURE

Synovial cells were isolated from the synovial membranes of the knees of Wistar rats. Following sequential pronase/collagenase digestion, synovial cells were isolated and adherent type B synoviocytes cultured as previously described[34]. The synovial fibroblasts were trypsinized and counted; 1/3 of the cells were transduced with Ad.GFAT/GFP, and 1/3 were transduced with Ad.GFP as a control for viral infection. The remaining 1/3 of the cells were not transduced. Unmodified, Ad.GFAT/GFP and Ad.GFP synoviocytes were then seeded at a density of 4×10^5 cells per well in 12 well plates and cultured in 1 ml of complete DMEM/Ham's F-12. Twenty-four hours later, co-cultures were established by addition of two alginate beads containing chondrocytes to each well. After 24 h, the co-cultures were challenged by the addition of human IL-1β (R&D system) at 0, 1, 5, or 10 ng/ml in 1 ml of complete medium. Twenty-four hours after addition of IL-1, the cultures were analyzed for evidence of cytokine stimulation.

HUMAN GFAT mRNA EXPRESSION

At approximately 90% confluence in 25-cm^2 flasks, individual cultures of rat synoviocytes were infected using a range of doses of Ad.GFAT/GFP (0 to 400 multiplicity of infection [MOI]) for 4 h in Gey's balanced solution (Life Technologies). Forty eight hours after transduction, total RNA was isolated from cell cultures by guanidinium thiocyanate; phenol:chloroform extraction using Trizol (Life Technologies) according to the manufacturer's protocol. The recovered RNA was quantified by spectrophotometry at 260/280 nm. The relative levels of GFAT and GAPDH mRNA were measured using RT-PCR. Total RNA was first

heated to 65°C for 10 min and placed on ice. Reverse transcription was performed in a final volume of 20 µl using 100 pmol of a hexamer random primer, 200 µM of each dNTP, 40 units of RNase OUT and 200 units of MML-V RNase H^- reverse transcriptase (Life technologies) (37°C 1 hr, 95°C 5 min). For PCR amplification, 2 µl of the RT reaction products were mixed in 50 µl with 200 µM of each dNTP, 20 pmol of sequence specific primers (MWG) and 2 units of Taq DNA polymerase (Life technologies). For amplification of human GFAT (32 cycles [45 s 95°C, 45 s 60°C, 45 s 72°C]), the forward primer extended from nucleotide 69 to 92, and the reverse primer was complementary to sequences at positions 877 to 900, in accordance with the human sequence (GenBank accession number M90516). To avoid quantitation of rat endogenous GFAT, amplification products were distinguished using restriction site polymorphism: a single recognition sequence for Bam HI in the rat GFAT amplification product permitted digestion into 2 fragments (253 and 613 bp), while the human product which does not contain this site remained uncut. For amplification of rat GAPDH (32 cycles [45 s 95°C, 45 s 65°C, 45 s 72°C]), the forward primer extended from nucleotides 758 to 781 and the reverse primer was complementary to nucleotides 983 to 1010, based upon the rat sequence (GenBank accession number AF106860). PCR products were then analysed during exponential phase of amplification. Following BamHI endonuclease digestion where appropriate, RT-PCR products were resolved by agarose gel electrophoresis (1.6%) and stained with ethidium bromide. DNA bands were visualized under UV light, photographed and quantitated by densitometric analysis with Scion Image software (NIH Image). After normalization of the RT-PCR products to GAPDH, GFAT levels were expressed relative to that observed at the lowest viral dose.

MEASUREMENT OF PROTEOGLYCAN SYNTHESIS

Following IL-1β treatment, alginate beads were removed from co-culture and incubated for 4 h in complete DMEM/Ham's F-12 medium supplemented with 10 µCi/ml of $Na_2^{35}SO_4$. The beads were then extensively washed with 0.15 M NaCl and solubilized overnight in Soluene-350 (0.5 M quaternary ammonium hydroxide in toluene). The amount of radiolabeled sulfate incorporated into newly synthesized glycosaminoglycans was quantitated by liquid scintillation counting[35,36].

NITRITE ASSAY

NO production was determined spectrophotometrically by measuring the accumulation of nitrite (NO^-_2), a stable breakdown product of NO, in harvested culture medium by the Griess reaction using sodium nitrite as a standard[37]. Briefly, 100 µl of culture supernatant were mixed with 100 µl of Griess reagent (sulfanilamide (1% [w/v]) in 2.5% H_3PO_4 and *N*-naphthylethylenediamine dihydrochoride (0.1% [w/v]) in H_2O) for 5 min in 96-well plates. The absorbance at 550 nm was measured on a MR5000 microplate reader. Nitrite concentration was calculated from a standard curve of sodium nitrite. Results are expressed in µM.

PGE_2 ASSAY

PGE_2 released by the chondrocytes and the synoviocytes was measured in conditioned media by specific ELISA according to the manufacturer's instructions (R&D System). This assay does not cross-react with other prostanoids. Results are expressed in ng/ml.

STATISTICAL ANALYSIS

After comparison of data by analysis of variance, different groups were compared using Fisher's *t*-test. Assays were made in triplicate. *P* values less than 0.05 were considered significant.

Results

GFAT TRANSGENE EXPRESSION IN TRANSDUCED RAT SYNOVIOCYTES

To determine a working viral dose for our subsequent experiments, individual cultures of rat synoviocytes were infected with a range of doses of Ad.GFAT/GFP spanning approximate multiplicities of infection (MOI) from 0 to 400. Because this vector enables simultaneous expression of both GFAT and GFP, we were able to visualize the percentage of cells transduced with each viral dose (Fig. 1A). This is especially important since there is no existing quantitative assay for GFAT protein. Human GFAT expression at the mRNA level, following transduction with various MOI of Ad.GFAT/GFP, was determined by semi-quantitative RT-PCR. For this, 48 h after infection, total RNA was extracted, and RT-PCR was performed to measure the expression of human GFAT mRNA at each viral dose relative to that of GAPDH (Fig. 1B, C). As shown in Fig. 1, from 100 to 400 MOI, nearly 100% of the synovial cells appeared to be transduced as shown by the number of fluorescent cells (Fig. 1A), and human GFAT mRNA expression reached its maximal value (Fig. 1B, 1D). All subsequent experiments were thus performed using a viral dose of 100 MOI of Ad.GFAT/GFP.

EFFECT OF Ad.GFAT/GFP TRANSDUCED SYNOVIOCYTES ON IL-1β MEDIATED INHIBITION OF CHONDROCYTE PROTEOGLYCAN SYNTHESIS

To test if overexpression of GFAT in synoviocytes can block the effects of IL-1 stimulation of chondrocytes in a three dimensional matrix, chondrocytes encapsulated in alginate beads were co-cultured for 24 h with 4×10^5 untransduced synoviocytes, or synoviocytes transduced with Ad.GFAT/GFP, or Ad.GFP as a control for viral transduction. Co-cultures were then challenged with 0, 1, 5 or 10 ng/ml IL-1β, and proteoglycan synthesis within the beads was measured 24 h later.

As expected, relative to control, IL-1β treatment of the cultures containing untransduced synoviocytes decreased proteoglycan synthesis of the chondrocytes in co-culture by 28% at 1 ng/ml, 36% at 5 ng/ml, and 44% at 10 ng/ml of the cytokine (Fig. 2). Similar reductions were observed in chondrocytes co-cultured with synoviocytes transduced with Ad.GFP. Remarkably, the inhibitory effect of IL-1β on proteoglycan synthesis in chondrocytes was fully blocked by co-culture with synoviocytes overexpressing GFAT, even at 10 ng/ml of IL-1β.

EFFECT OF Ad.GFAT/GFP TRANSDUCED SYNOVIOCYTES ON IL-1β MEDIATED STIMULATION OF NO AND PGE_2 SYNTHESIS

To investigate further the influence of GFAT overexpression in synovial fibroblasts on the effects of IL-1β in

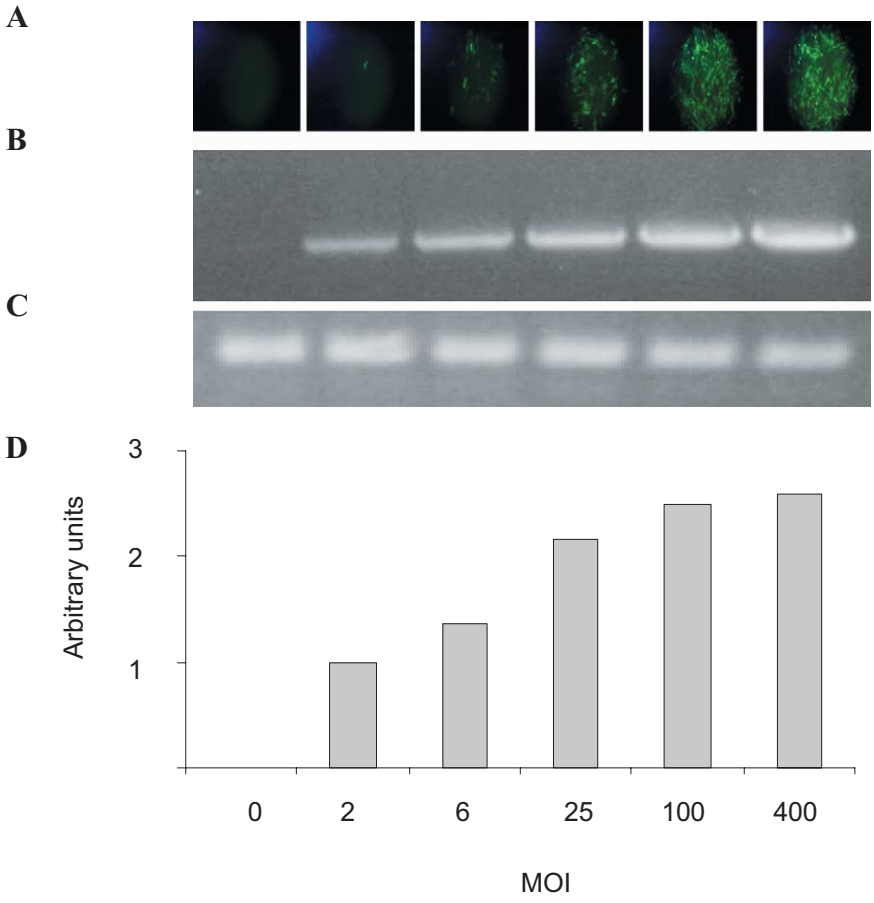

Fig. 1. Transgene expression in synovial fibroblast following transduction with Ad.GFAT/GFP**.** Primary cultures of synovial fibroblasts were grown to confluence in 25-cm^2 flasks and infected with different doses of an adenovirus carrying cDNA for both GFAT and GFP. Two days after infection, GFP expression was visualized under fluorescent microscopy (A), total RNA was then extracted and GFAT and GAPDH mRNA expression were analyzed by RT-PCR. Electrophoretic profiles of GFAT (B) and GAPDH (C) RT-PCR products for each dose of adenovirus are shown. Using scanning densitometry analysis of RT-PCR products illustrated in (B) and (C), respective GFAT products were normalized to GAPDH. The levels of GFAT products were then plotted relative to that obtained at an MOI of 2 (D).

the co-cultures, we examined the NO and PGE_2 levels in the conditioned media from the previous experiments. In the absence of IL-1β treatment, the untransduced and adenoviraly infected co-culture systems showed similar baseline levels of NO production of approximately 4 μM (Fig. 3A). In the co-cultures with unmodified synovial fibroblasts and those transduced with Ad.GFP, NO levels were found to increase with the amount of IL-1β added and at highest dose reached a maximum of 21 and 20 μM, respectively. Interestingly, co-culture of chondrocytes with synovial fibroblasts transduced to overexpress GFAT strongly reduced NO production in response to IL-1β. Indeed, full inhibition was observed when cells were challenged with the lowest dose of IL-1β and only a slight increase to 5.5 and 7 μM was detected in the presence of 5 and 10 ng/ml of IL-1β respectively.

PGE_2 levels in the conditioned media were measured by specific ELISA. As shown in Fig. 3B, IL-1β strongly increased PGE_2 production when the chondrocyte beads were co-cultured with normal synoviocytes (up to 850 ng/ml for 10 ng/ml of IL-1β) or Ad.GFP transduced synoviocytes (up to 767 ng/ml at 10 ng/ml of IL-1β). However, the presence of synovial cells overexpressing GFAT reduced PGE_2 production at all doses of IL-1β. The highest dose of IL-1β produced 163 ng/ml of PGE_2, corresponding to an 80% decrease vs co-culture using normal synoviocytes.

To provide an indication of the proportion of NO and PGE_2 produced by each cell type in the co-culture system, chondrocyte beads or untransduced synoviocytes were cultured separately. NO and PGE_2 levels were then measured in media conditioned by the respective cultures with or without IL-1β challenge at 10 ng/ml. As shown in Fig. 4A, following IL-1 stimulation, chondrocytes produced 5.5 μM and synoviocytes 12.33 μM of NO, which represent 26 and 59%, respectively of the total NO production in a co-culture system (21 μM). Analyses of the same conditioned media also showed that chondrocytes produced 148 ng/ml and synovial fibroblasts 622 ng/ml of PGE_2, which represent 17% and 73%, respectively of the total PGE_2 production in a co-culture system (850 ng/ml) (Fig. 4B).

Discussion

In this study, synovial fibroblasts were genetically modified with an adenovirus carrying the cDNA for GFAT and then were co-cultured with chondrocytes encapsulated in

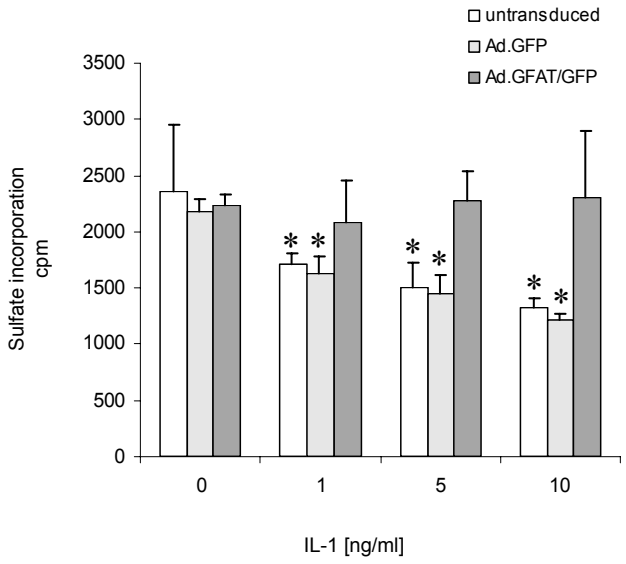

Fig. 2. Effect of Ad.GFAT/GFP transduced synoviocytes on IL-1β mediated inhibition of chondrocyte proteoglycan synthesis. Chondrocytes encapsulated in alginate beads were co-cultured with untransduced synovial fibroblasts or those infected with either Ad.GFAT/GFP or Ad.GFP in complete medium for 24 h. IL-1β was then added to individual culture at 0 ng/ml; 1 ng/ml; 5 ng/ml; or 10 ng/ml. Twenty-four hours later, proteoglycan synthesis was evaluated by incorporation of radiolabeled sodium sulfate. Results are expressed in cpm (each bar represents the mean of three assays. Error bars represent one S.D.). *, $P<0.05$ versus untransduced control by Fisher's *t*-test.

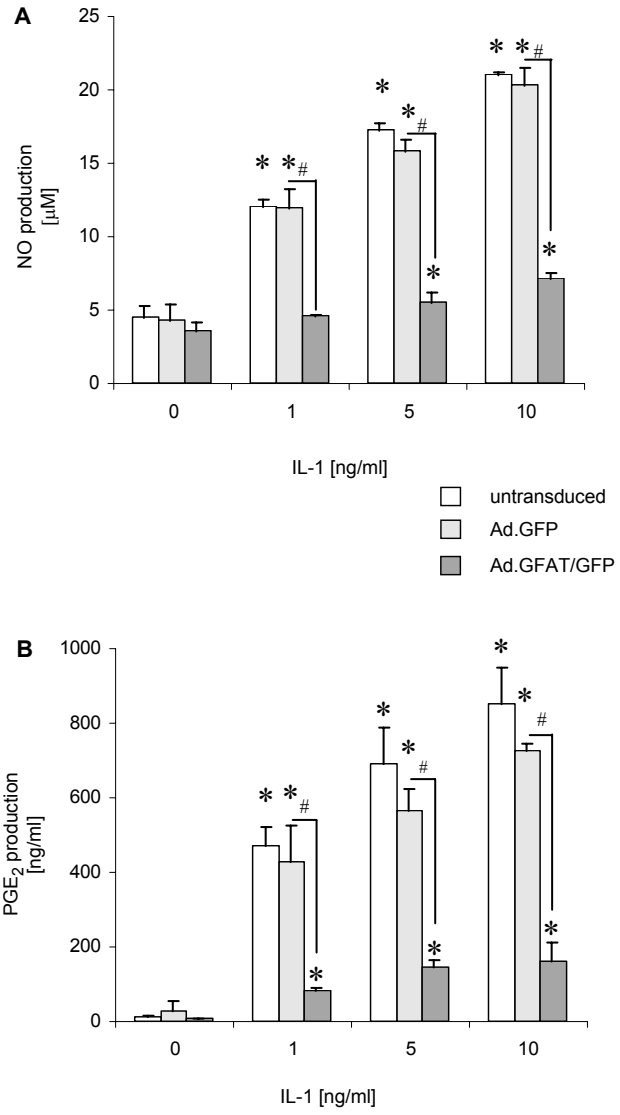

Fig. 3. Effect of GFAT overexpression on IL-1β mediated stimulation of NO and PGE_2 production. Chondrocytes encapsulated in alginate beads were co-cultured for 24 h with untransduced synovial fibroblasts or those infected with either Ad.GFAT/GFP or Ad.GFP. IL-1β was then added to individual cultures at 0; 1; 5; or 10 ng/ml. Twenty-four hours later, NO production was assessed in the conditioned media by measurement of nitrite (A). Results are expressed in μM nitrite. PGE_2 concentrations (B) were also determined by ELISA in the medium. Results are expressed in ng/ml PGE_2 Each bar represents the mean of three assays. Error bars represent one S.D. *, $P<0.05$ vs untransduced control, #, $P<0.05$ versus Ad.GFP control by Fisher's *t*-test.

alginate beads. Since the primary site of gene transfer following direct intra-articular injection of a recombinant vector occurs in the synovial lining, this co-culture system was designed to test the practical application of GFAT gene delivery by simulating the physical separation of the chondrocytes sequestered in cartilage matrix from transduced synoviocytes *in vivo*. In this *in vitro* system, overexpression of GFAT in synoviocytes antagonized the effects of IL-1β on the co-culture system. The level of inhibition was similar to that observed when chondrocytes were cultured in the presence of high concentrations (20 mM) of exogenous glucosamine[17]. Indeed, as well as relieving the inhibition of proteoglycan synthesis in chondrocytes, overexpression of GFAT in synovial cells almost totally suppressed IL-1β-mediated increases in NO and PGE_2 production, suggesting an inhibition of their synthesis by both synovial fibroblasts and chondrocytes in the co-culture system.

Although our results do not identify the biological mechanism involved in the protection against IL-1, they implicate involvement of a soluble mediator. GFAT is a key enzyme in the hexosamine pathway, which within the cell catalyzes the synthesis of glucosamine and other precursors of macromolecules based on this amino-sugar. Thus, although the genetically modified synovial cells may have increased levels of intracellular glucosamine-6-phosphate, the production of other glucosamine derivatives may also be increased. It is therefore possible that GFAT overexpression in synoviocytes is capable of activating multiple protective pathways in responsive cells. Our previous data[18] identifying nuclear factor-κB activation as a target of glucosamine action are consistent with this suggestion. In the present system, IL-1β stimulation of chondrocytes was blocked by exposure to synoviocytes genetically modified to overexpress GFAT. This suggests that glucosamine and/or downstream cellular products arising from overproduction of GFAT would have first to diffuse from the modified synoviocytes to inhibit the response of the chondrocytes to IL-1β. Specific experiments to elucidate the mechanism are currently underway.

In vitro, glucosamine-mediated inhibition of IL-1 required high concentrations that may be unachievable by oral administration, as most glucosamine formulations do not surpass 1.5 g per day. An additional disadvantage of the

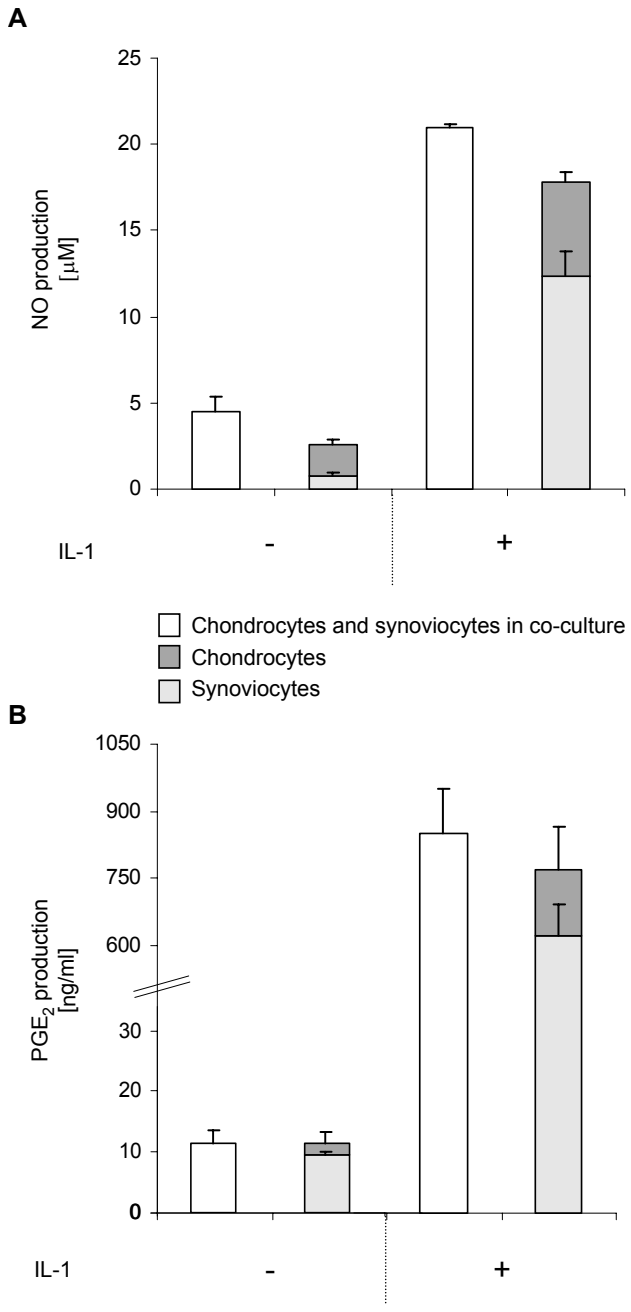

Fig. 4. Comparison of IL-1β-induced NO and PGE_2 production from synoviocytes and chondrocyte beads individually or in co-culture. Untransduced synovial fibroblasts and chondrocyte beads were cultured individually for 24 h prior to IL-1β stimulation (10 ng/ml). Twenty-four hours after the addition of IL-1, NO production was assessed by measurement of nitrite in the conditioned media (A). Results are expressed in μM nitrite. PGE_2 concentrations in the medium (B) were also determined by ELISA. Results are expressed in ng/ml PGE_2. (Each bar represents the mean of three assays. Error bars represent one S.D.).

oral administration of glucosamine is the possibility of inducing insulin resistance and other systemic effects. Intra-articular gene delivery may offer one strategy for circumventing this problem. Indeed, by limiting gene transfer to diseased joints, exposure of extra-articular tissues to the effects of GFAT overexpression would be minimized. Data from our laboratory have shown that the synovial lining is capable of maintaining therapeutic levels of transgene expression for several months[39], providing optimism that the use of GFAT gene transfer might be effective in the treatment of chronic articular disease such as OA. An appealing aspect to a gene delivery approach such as this, is that the gene product delivered has no known immunomodulatory activities, which may reduce potential side effects, such as increased susceptibility to infection in contrast to TNF receptors, IL-4 or vIL-10[25,38–44]. Nevertheless, the effect of long term overproduction of GFAT on the biology of the synovial lining cells remains unknown.

In conclusion, the results of the present study demonstrate the feasibility of antagonizing the response of chondrocytes to IL-1β by transfer of GFAT cDNA to synovial fibroblasts *in vitro* and lay the foundation for studies in animal model to explore the merit of therapeutic application in OA.

Acknowledgements

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